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Comprehensive analysis of gene mutation and expression profiles in neuroendocrine carcinomas of the stomach

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ABSTRACT

The gene mutation and expression profiles of gastric neuroendocrine carcinoma (NEC) have not been comprehensively determined. Here, we examined the gene mutation and expression profiles of NEC using whole exome sequencing (WES) and microarray analysis. Six patients with gastric NEC and 13 with gastric adenocarcinoma (GAD) were included in this study. Single nucleotide variants were compared and multivariate statistical investigation with orthogonal partial least squares discriminant analysis (OPLS-DA) was performed to compare the difference in expression profiles between NEC and GAD. NEC showed a significantly higher mutation rate than GAD and the percentage difference in the mutation pattern of NEC compared with GAD was 92.8%. OPLS-DA clearly discriminated between NEC and GAD. We identified 35 genes, including CPLX2 (Complexin 2), which were expressed more strongly in NEC than in GAD, of which 14 were neural-related. Immunohistochemical analysis showed the strong expression of CPLX2 in all NECs, versus expression in only 2 of 13 GADs. Gastric NEC had a specific mutation pattern with a significantly higher gene mutation rate than GAD, and completely differed from GAD on the basis of gene expression profile. CPLX2 might be a potential novel biomarker for the diagnosis of NEC.

Neuroendocrine carcinoma (NEC) of the stomach is a rare disease, accounting for only 0.4% to 0.6% of all gastric cancers. Although NEC is categorized as a neuroendocrine neoplasm, it is clearly differentiated from other neuroendocrine tumors by its highly malignant biological behavior (24). In addition, gastric NEC is considered to have a poorer prognosis than gastric adenocarcinoma (GAD), with a high incidence of metastasis to the lymph nodes or liver in the early stages of the disease (12, 16). However, as gastric NEC is morphologically similar to poorly differentiated adenocarcinoma with a predominantly solid growth pattern (12), it is difficult to distinguish using hematoxylin and eosin staining only. Although immunohistochemistry for neuroendocrine markers such as chromogranin A (CGA), synaptophysin (SYP) and neural cell adhesion molecule (NCAM) is widely used for diagnosis (5, 24), these markers are not expressed in all cases of gastric NEC, which sometimes hinders diagnosis.

Gastric cancer is a heterogeneous disease and the presence of a specific oncogene or tumor suppressor

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gene mutation is rare. TP53 is the most frequently mutated gene, occurring in 29–55% of gastric cancers (2, 3, 8), followed by PTEN, ARID1, APC, CDH1, MUC6, CTNNA2, CLI3, RNF43, and RHOA (28, 29). Meanwhile, there are distinct tumors which show hypermutation, mainly caused by deficient DNA mismatch repair genes, which are found in 13–14% of gastric cancers (22). Recently, two novel molecular profiling-based classifications for gastric cancer have been proposed (1, 4). These studies identified key driver genes that were enriched in a specific molecular subgroup. However, because gastric NEC is so rare, few studies have reported the genetic features of these tumors.

Here, to identify novel, clinically useful biomarkers for the diagnosis of gastric NEC, we performed comprehensive gene mutation and expression analyses of gastric NEC.

MATERIALS AND METHODS

Subjects. Surgically resected tumor specimens and corresponding peripheral blood samples were obtained from 104 consecutive patients who underwent gastrectomy at the Division of Gastric Surgery of Shizuoka Cancer Center between January 2014 and March 2015 and had enrolled in Project HOPE (High-tech Omics-based Patient Evaluation), a study launched at Shizuoka Cancer Center with the aim of evaluating the biological characteristics of cancer and diathesis of each patient by multiomics-based analyses (31). All pathological slides of specimens from those patients were reviewed, and seven patients whose resected samples were proven to contain NEC cells without adenocarcinoma cells were selected. Fifteen patients with GAD were randomly selected as the control. Previous reports indicated that tumors with microsatellite instability (MSI) are distinct biological entities (1, 29) and that the presence of MSI may significantly affect gene mutation analysis. On the basis of one of these studies (1), we considered that MSI was present when tumors had more than 500 mutations per tumor and low MLH1 (MutL homolog 1) expression, and excluded these cases from analysis. One NEC and two GADs matched the criteria, leaving a total of 19 patients (6 with NEC and 13 with GAD) for inclusion in this study. Clinical and pathological data were collected from our prospectively recorded database. The Japanese Gastric Cancer Association classification system was used to classify the macroscopic tumor type, histological tumor type, tumor depth (T), lymph node status (N), distant metastasis (M) and pathological stage (13).

The research plan was designed according to the revised Ethical Guidelines for Human Genome/Gene Analysis Research in Japan (http://www.lifescience.mext.go.jp/files/pdf/n1115_01.pdf) and the study protocol was approved by the Institutional Review Board of Shizuoka Cancer Center (approval number #25-33). Written informed consent was obtained from all participants.

Clinical samples. We acquired blood samples from patients and paired them with the corresponding resected, flash-frozen tissue samples from the HOPE study. DNA was extracted from blood and tissues by the same method using a QIAamp DNA Blood Kit (QIAGEN, Hilden, Germany), except that tissues were treated with Proteinase K (QIAGEN) (21). A board-certified pathologist determined that tumor purity was > 50%. DNA was quantified using Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA) and Qubit 2.0 Fluorometer (Thermo Fisher Scientific).

Next-generation sequencing. The exome library used for WES was prepared using an Ion Torrent AmpliSeq RDY Exome Kit (Thermo Fisher Scientific) in accordance with the manufacturer's recommended protocol. A total of 100 ng of DNA was used for target amplification under the following conditions: 99°C for 2 min, followed by 10 cycles at 95°C for 15 s and 60°C for 16 min, and a final hold at 10°C. Incorporated primer sequences were partially digested using a proprietary method. Ion Torrent Proton adapters were ligated to the amplicons at 22°C for 30 min followed by 72°C for 10 min, and the library was purified with Agencourt Ampure XT beads (Thermo Fisher Scientific). Libraries were quantified using a quantitative polymerase chain reaction (qPCR), and DNA (8 pM) was sequenced using the semiconductor DNA sequencer (Ion Torrent Proton Sequencer, Thermo Fisher Scientific) according to the manufacturer's protocol. Matched tumor–normal pair somatic variants were identified using Ion Reporter ver. 4.4 software (Thermo Fisher Scientific) (11) after base calling, quality trimming, and mapping to the hg19/GRCh37 reference genome using Torrent Suite software ver. 4.4 (Thermo Fisher Scientific) (26). In this step, sequence data derived from tumor and blood samples were analyzed separately, and the latter were used as matched controls. In this process, only somatic variants remain after the subtraction of variants from blood data from the variants acquired from tumor data. In this variant-call

workflow, we identified somatic mutations that satisfied the thresholds quality score ≥ 60 or depth of coverage ≥ 20. Somatic variants were manually inspected using the Integrative Genomics Viewer (19). Annotation of detected SNVs was performed using databases that included germline and somatic variants, as follows: COSMIC (15), ClinVar (17), dbSNP (23), UniProt (27) and DrugBank (30). In the present study, we focused our analyses on nonsynonymous single nucleotide variants (SNVs) located in an exon or splice site.

Comprehensive gene expression analysis using DNA microarray. Fresh tumor and adjacent normal tissues were soaked in RNAlater reagent (Thermo Fisher Scientific), and total RNA was isolated and purified using a miRNeasy Mini Kit (QIAGEN) according to the manufacturer's protocol. RNA quality was evaluated by RNA integrity number, which was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA samples with an RNA integrity number ≥ 6.0 were used for gene expression analysis.

Gene expression analysis was performed on a SurePrint G3 Human Gene Expression 8×60K v2 Microarray (Agilent Technologies) using a One-color Low Input Quick Amp Labeling kit (Agilent Technologies) according to the manufacturer's instructions. Data processing to generate raw signal intensity data was performed with GeneSpring version 13.1.1 software (Agilent Technologies). The raw signals were log-transformed and normalized (GeneSpring software, Agilent Technologies) and the difference in the normalized microarray signal intensities (fold change) between the tumor and adjacent normal tissues were calculated.

Immunohistochemical analysis. Routine pathological diagnosis was done using surgically resected tumors fixed in 10% formalin and embedded in paraffin. Paraffin sections of 3 μm thickness containing representative histology of the tumor were used for immunohistochemical analysis. Immunohistochemical staining was performed using the Bond III automated stainer and BOND Polymer Refine Detection kit (Leica Biosystems, Bannockburn, IL). The sections were pretreated with epitope retrieval BOND1 for 20 min at 100°C and then reacted with the primary antibodies. After reaction with diaminobenzidine chromogen, the sections were counterstained with hematoxylin, and the stained sections were independently evaluated by two investigators (R.M. and T.N.). The staining for all the antibodies were classified into two categories according to the percentage of positive cells: staining in ≥ 10% of tumor cells was regarded as positive, and in < 10% of cells as negative.

To confirm the neuroendocrine features of the tumor, the expression of CGA, SYP and NCAM was studied using immunohistochemical methods that were applied in routine pathological diagnosis.

Statistical analysis. Statistical analyses were performed using Fisher's exact test and the Mann–Whitney U test, using R statistics version 3.2.2. Statistical significance was defined as $P < 0.05$.

To compare the difference in expression profile between NEC and GAD, multivariate statistical investigation with orthogonal partial least squares discriminant analysis (OPLS-DA) was performed with the help of SIMCA-P+ software (v 12.0.1.0; Umetrics, Umea, Sweden) using microarray data. The quality of the OPLS-DA model was evaluated by the explained parameter (R^2) and the predictive parameter (Q^2). Values of 0.5 indicated an acceptable OPLS-DA model (6). S-plot p (corr) values > 0.8 or < −0.8 resulting from OPLS-DA were considered to have strongly contributed to the differences between the two histological types.

RESULTS

Patient characteristics

Patient characteristics are shown in the Table 1. There were no significant differences in patient characteristics between the NEC and GAD groups, including T, N and M staging.

Gene mutation profiling

Gene mutation profiling of NEC and GAD is shown in Fig. 1. A total of 588 nonsynonymous somatic mutations affecting 557 genes were identified in the six NECs. There was an average of 98.0 ± 32.7 somatic mutations (synonymous and nonsynonymous) per NEC tumor, which was significantly higher than the average 62.5 ± 75.7 mutations per tumor in GAD ($P = 0.023$). The most frequently mutated gene was TP53 (100%) in NEC, whereas this was observed in only 6 of 13 GADs (46%) cases. The TP53 mutation rate was significantly higher in NEC than in GAD ($P = 0.044$).

Of the 557 genes mutated in NEC, 40 genes (7.2%) were mutated in both NEC and GAD, while 517 genes (92.8%) were mutated only in NEC. These 517 genes mutated only in NEC included many neural-related genes, such as TSHZ3 (Teashirt Zinc Fin-

Table 1 *Patient characteristics*

		NEC (n = 6)	GAD (n = 13)
Sex	Male	4	8
	Female	2	5
Age (years)	(Median)	67	72
Tumor diameter (mm)	(Median)	50	70
Macroscopic type	Type 1	1	2
	Type 2	5	4
	Type 3	0	7
	Type 4	0	0
Tumor depth	T1	1	0
	T2	1	4
	T3	2	3
	T4	2	6
Lymph node status	N0	2	4
	N1	2	3
	N2	1	0
	N3	1	6
Distant metastasis	M0	5	12
	M1	1	1
Stage	I	1	1
	II	3	5
	III	1	6
	IV	1	1

ger Homeobox 3), SEMA5A (Semaphorin 5A), TPH2 (Tryptophan Hydroxylase 2), SDK1 (Sidekick Cell Adhesion Molecule 1), and PLXNA1 (Plexin A1). We identified 20 genes that were mutated in two NECs, of which 4 were cancer-associated genes, namely SYNE1 (Spectrin Repeat Containing Nuclear Envelope Protein 1), TSHZ3, LRP1B (LDL Receptor Related Protein 1B), and MECOM (MDS1 And EVI1 Complex Locus). The SYNE1 gene mutation was also observed in GAD, but other mutations were observed only in NEC. A total of 536 genes, including SMAD4 (SMAD Family Member 4), PIK3CA (Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha), RB1 (Retinoblastoma 1) and KRAS (Kirsten Rat Sarcoma Viral Oncogene Homolog), were mutated in only 1 NEC tumor.

Comparison of expression profile between NEC and GAD with multivariate statistical investigation

Subsequent gene expression analysis using DNA microarray was performed, and OPLS-DA was performed to examine the difference in gene expression profiles between gastric NEC and GAD.

The OPLS-DA score plot is shown in Fig. 2A. A clear distinction between NEC and GAD was seen.

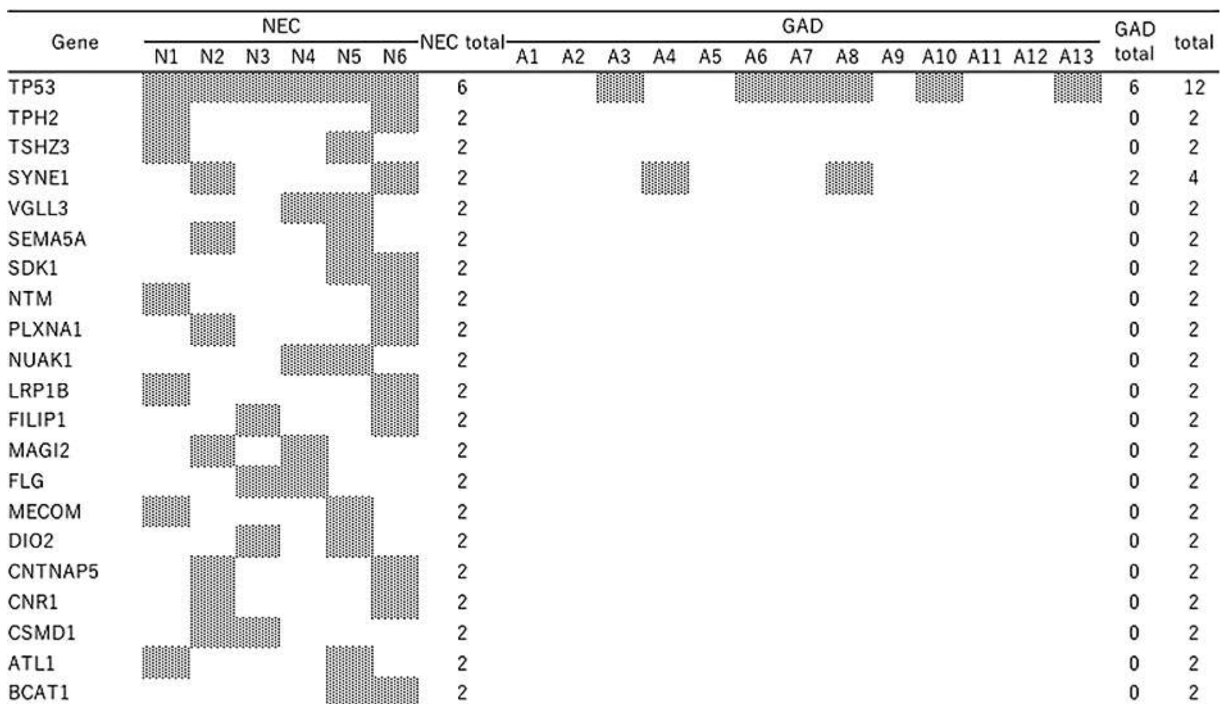

Fig. 1 Profiling of gene mutations in gastric neuroendocrine carcinoma (NEC) and adenocarcinoma (GAD). The vertical axis shows the name of the mutated genes, and the horizontal axis shows the patients. Mutated genes in at least two patients with NEC are shown.

The explained parameter R^2 and the predictive ability Q^2 were 0.996 and 0.580, respectively, indicating the reliability of the OPLS-DA model. The OPLS-DA loading S-plot, a plot of covariance versus correlation in conjunction with the variable trend plots, allows for easier identification of significant genes that contribute to the differences between NEC and GAD (Fig. 2B). As a result, 44 genes with p (corr) > 0.8 or < −0.8 were selected, and were considered to have strongly contributed to the differences between NEC and GAD (Supplemental figure).

Among these 44 genes, information on 35 genes with p (corr) > 0.8 that were expressed more strongly in NEC than in GAD is shown in Table 2. Of these 35 genes, 14 genes (40%), including MYT1 (Myelin Transcription Factor 1), CPLX2 (Complexin 2), SLC36A4 (Solute Carrier Family 36 Member 4), HIP1 (Huntingtin Interacting Protein 1), SYP and PROX1 (Prospero Homeobox 1), were related to neurogenesis or were expressed in neurons. Information on nine genes with p (corr) < −0.8 that were expressed more strongly in GAD than in NEC is shown in the Table 3.

Immunohistochemical analysis

We next performed immunohistochemical analyses to confirm the protein expression of these genes in NEC and GAD. Among the 35 genes with p (corr) > 0.8, 5 genes (MYT1, CPLX2, SLC36A4, HIP1, and PROX1) that are reportedly related to the nervous system were selected.

The results of immunohistochemical analysis are shown in Table 4. CPLX2 was strongly expressed in the cytoplasm in all NECs, but was positive in only 2 of 13 GADs. Immunohistochemical analysis of expression of CPLX2 and common neuroendocrine markers (CGA, SYP and NCAM) in NEC are shown in Fig. 3. SLC36A4 was positive in 5 cases (83%) of NEC and 11 cases of GAD (84%). HIP1 was positive in all NECs and GADs. MYT1 and PROX1 were not expressed in either NEC or GAD.

The results of immunohistochemical analysis of other neuroendocrine markers in NEC were as follows: CGA was positive in 4 cases (67%), SYP was positive in all 6 cases, and NCAM was positive in 5 cases (83%).

DISCUSSION

This comprehensive molecular analysis of gastric NEC produced two important findings. First, we examined the gene mutation profile in gastric NEC using next generation sequencing (NGS) and demonstrated that the somatic mutation rate was significantly higher in NEC than in GAD. In particular, TP53 mutation was more frequently observed in NEC than in GAD, and genes related to the nervous system were frequently mutated in NEC. Second, we showed that gastric NEC and GAD were completely different tumors based on gene expression profiling and that 40% of the genes expressed strongly in NEC were related to the nervous system. In addition,

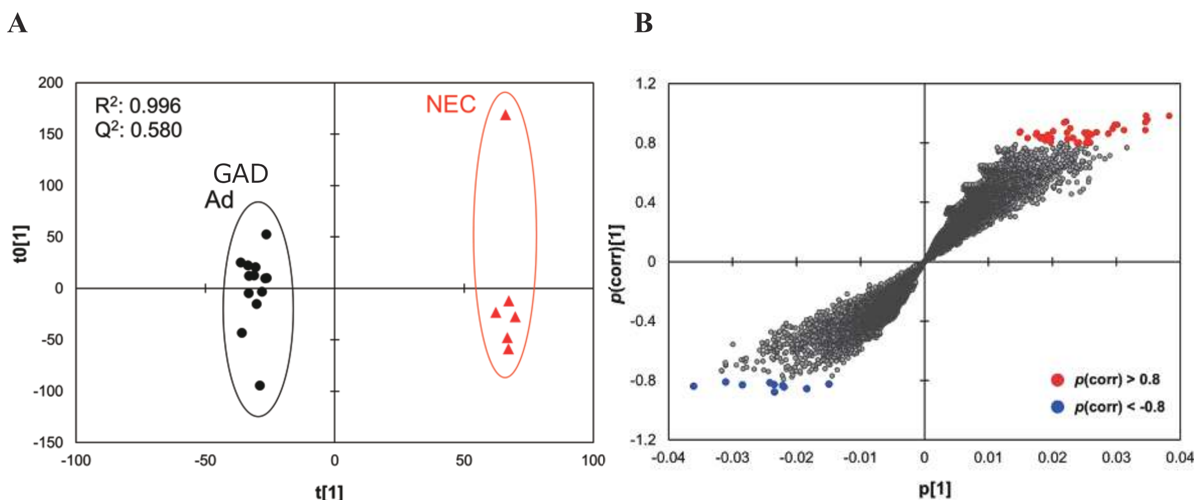

Fig. 2 Results of multivariate statistical investigation by orthogonal partial least squares discriminant analysis (OPLS-DA). **A**: The OPLS-DA score plot shows two significant components corresponding to NEC and GAD. **B**: Identification of genes contributing to the difference between both histological types based on the s-plot values resulting from OPLS-DA. A total of 44 genes (listed in Fig. 3) indicated by red and blue spheres with p (corr) > 0.8 or p (corr) < –0.8 were selected and considered to have strongly contributed to the differences between NEC and GAD.

Table 2 *Information on 35 genes showing p (corr) value > 0.8*

Gene symbol	Average concentration (Log$_2$ ratio)		OPSL-DA
	NEC	GAD	S-Plot p (corr) value
MYT1	5.52	0	0.98
CHRNB2	5.87	0.1	0.96
ZNF775	2.41	0.08	0.94
DRD2	6.02	0.27	0.94
AP3B2	4.68	0.23	0.92
CPLX2	4.31	0	0.92
LOC284669	2.84	0.26	0.9
KCNH6	4.38	0	0.9
VWA5B2	2.92	−2.03	0.89
STRA6	2.92	0.32	0.89
BUB3	2.92	0	0.88
GOLGA7B	2.92	0.51	0.87
RUNDC3A	2.92	0.32	0.87
TMEM198	2.92	0.06	0.87
SMC3	2.92	0	0.87
BSN	2.92	0.08	0.87
SLC36A4	2.92	0.19	0.87
NOL4L	2.92	0	0.86
LOC100128563	2.92	0.08	0.86
AMER3	2.92	0	0.84
EXOC7	2.92	0.24	0.84
CAMK2N2	2.92	−0.12	0.84
SMYD3	2.92	−0.04	0.84
TMEM178B	2.92	0.17	0.84
HIP1	2.92	0	0.83
SRRM3	2.92	0.37	0.83
SYP	2.92	0	0.83
ITPR2	2.92	0	0.83
SBK1	2.92	0.08	0.83
CDKAL1	2.92	0	0.82
SCN8A	2.92	0.11	0.81
PROX1	2.92	0.57	0.8
OVOS2	2.92	0.56	0.8
TEX101	2.92	0	0.8
EVA1A	2.92	0.59	0.8

Table 3 *Information on nine genes showing p (corr) value < −0.8*

Gene symbol	Average concentration (Log$_2$ ratio)		OPSL-DA
	NEC	GAD	S-plot p (corr) value
HOXA13	0.36	5.75	−0.81
CEBPA	−2.45	0.8	−0.82
MPPE1	−1.13	0.09	−0.82
ACSM3	−4.94	−0.62	−0.3
KCTD1	−2.33	0.3	−0.83
ST6GALNAC1	−8.46	−1.48	−0.84
MOB3B	−2.82	−0.28	−0.84
NFIB	−1.82	−0.02	−0.85
TMEM37	−3.12	−0.32	−0.88

Table 4 *Antibodies used*

Antibody	NEC	GAD
MYT1	0 (0%)	0 (0%)
CPLX2	6 (100%)	2 (15%)
SLC36A4	5 (83%)	11 (85%)
HIP1	6 (100%)	13 (100%)
PROX1	0 (0%)	0 (0%)

the results suggest that CPLX2 is a potential novel biomarker for the immunohistochemical diagnosis of gastric NEC. This is the first report to comprehensively evaluated gene mutations and mRNA expression in gastric NEC.

First, we demonstrated that the average somatic mutation rate was significantly higher in NEC than in GAD. The most frequently mutated gene in NEC was TP53 (100%), which was more frequently mutated in NEC than in GAD ($P = 0.044$). Although there is a lack of information on TP53 gene mutation in gastric NEC, a few studies have investigated TP53 genetic alteration in gastric mixed adenoneuroendocrine carcinoma (MANEC). Scardoni *et al.* (20) observed TP53 gene mutation in 91.7% of neuroendocrine and adenocarcinoma components in MANEC of the gastrointestinal tract, including two gastric MANECs, while Nishikura *et al.* (18) observed TP53 gene mutation in 8/15 (53.3%) patients with gastric MANEC.

We identified 557 nonsynonymous gene mutations, including those of 92 cancer-associated genes, in NEC in this study. Because of the rarity of gastric NEC, few studies have investigated the genetic alterations in this tumor. Scardoni *et al.* (20) reported somatic mutations of ERBB1, ATM and RB1 in the neuroendocrine component of the stomach, but investigated only 54 cancer-associated genes. In the present study, we investigated more than 800 cancer-associated genes, and consider that our results provide greater detail. Interestingly, the majority of genes (517/557, 92.8%) mutated in NEC were not mutated in GAD, and included many neural-related genes, such as TSHZ3 and SEMA5A. These findings endorse the notion that NEC is a genetically different tumor from GAD. These mutations may be associated with the tumorigenesis and development of gastric NEC, and further study will be worthwhile.

NEC is morphologically similar to poorly differentiated adenocarcinoma with a predominantly solid

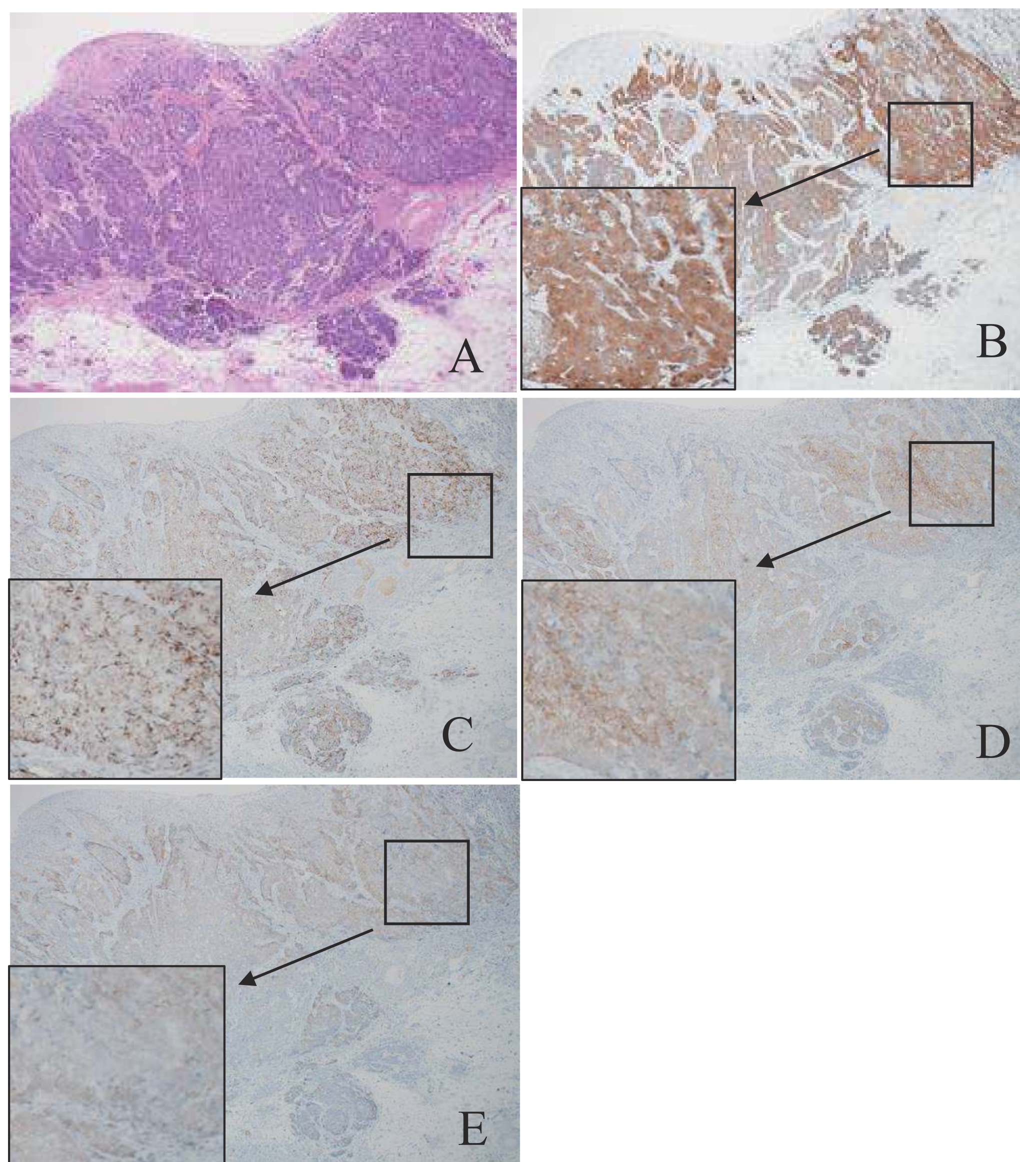

Fig. 3 (**A**) Hematoxylin and eosin staining of gastric NEC. (**B**) Immunohistochemical analysis of CPLX2 in gastric NEC, which showed strong expression in the cytoplasm. (**C**) Immunohistochemical analysis of CGA expression. (**D**) Immunohistochemical analysis of SYP. (**E**) Immunohistochemical analysis of NCAM. CPLX2: complexin-2; CGA: chromogranin A; SYP: synaptophysin; NCAM: neural cell adhesion molecule

growth pattern (12). This similarity hinders their differentiation in the absence of immunohistochemistry to identify neuroendocrine markers. Based on gene expression profiling using OPLS-DA analysis, however, we show here that gastric NEC and GAD are completely different tumors. The frequent inclusion of adenocarcinoma components in gastric NECs is well known. The latest WHO classification for neuroendocrine neoplasms distinguishes NEC from MANEC based on the proportion of NEC and adenocarcinoma components: a tumor with an NEC component of more than 70% is defined as NEC while a tumor with an NEC component of 30–70% is defined as MANEC (24). Previous studies have reported that both NEC and adenocarcinoma components of gastric MANEC have a common genetic aberration; thus, both components are considered to have a common origin (7, 18). In the present study, we found several gene mutations that were present in both NEC and GAD, although these tumors were obviously different on the basis of gene expression profile. These findings suggest that although the gene

mutation profiles of NEC, MANEC and AD share some similarities, changes in their gene expression profiles occur during the differentiation process.

In the gene expression analysis, we demonstrated that 35 genes were expressed at a significantly higher level in NEC than in GAD. The fact that 14 of these 35 genes were neural-related to some extent supports the concept that NEC has characteristics of neural tissue. Among the 35 genes, we chose 5 that have been reported to be related to cancer or development of the nervous system for immunohistochemistry. Of these 5, CPLX2 was expressed in all gastric NECs but in only 2 of 13 (15%) GADs, whereas the other 4 genes showed no difference between the tumors. CPLX2, a member of the complexin/synaphin family, is a soluble pre-synaptic protein believed to be involved in synaptogenesis and the regulation of neurotransmitter release from pre-synaptic terminals in the mature brain (9, 25). Previous studies have reported that CPLX2 plays a key role in maintaining normal neurological function, while its downregulation changes the neurotransmitter release that is sufficient to cause significant behavioral abnormalities, such as depression (10). Komatsu *et al.* (14) reported that CPLX2 was strongly positive in 16.3% of large cell neuroendocrine carcinomas of the lung and small cell carcinomas of the lung, but completely negative in all adjacent non-cancerous tissues and in non-small cell lung carcinomas. They also demonstrated that positive CPLX2 expression was associated with lymph vessel invasion ($P = 0.016$), pathological stage ($P = 0.031$) and poor disease-specific survival ($P = 0.004$) in these patients.

Although CGA, SYP, and NCAM are the main neuroendocrine makers for detecting neuroendocrine differentiation, these neuroendocrine markers are not expressed in all gastric NEC. Ishida *et al.* (12) reported positive rates of CGA, SYP, and NCAM of 86%, 94%, and 64%, respectively. Although our sample size was small, CPLX2 was overexpressed in all NECs, which suggests that CPLX2 might be a novel biomarker for the diagnosis of gastric NEC.

The present study included only six NECs, which might not be sufficient to provide a comprehensive view of the gene mutation and expression profiles of NEC. Nevertheless, no comprehensive genomic analysis of NEC yet exists, and our present findings may remain the only available information until a larger study is reported. Because we did not perform a pathway analysis, the relationships between the genes expressed in NEC are not clear.

In conclusion, we demonstrated that gastric NEC had a specific mutation pattern with a significantly higher gene mutation rate than GAD, and completely differed from GAD on the basis of gene expression profile. In addition, CPLX2 was exclusively expressed in NEC, as demonstrated by immunohistochemistry, and might be a potential novel biomarker for the diagnosis of NEC.

CONFLICT OF INTEREST

The authors have no competing interest to declare.

REFERENCES

1. Cancer Genome Atlas Research Network (2014) Comprehensive molecular characterization of gastric adenocarcinoma. *Nature* **513**, 202–209.
2. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E, Antipin Y, Reva B, Goldberg AP, Sander C and Schultz N (2012) The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov* **2**, 401–404.
3. COSMIC, Catalogue of Somatic Mutations in Cancer (URL: http://cancer.sanger.ac.uk/wgs)
4. Cristescu R, Lee J, Nebozhyn M, Kim KM, Ting JC, Wong SS, Liu J, Yue YG, Wang J, Yu K, Ye XS, Do IG, Liu S, Gong L, Fu J, Jin JG, Choi MG, Sohn TS, Lee JH, Bae JM, Kim ST, Park SH, Sohn I, Jung SH, Tan P, Chen R, Hardwick J, Kang WK, Ayers M, Hongyue D, Reinhard C, Loboda A, Kim S and Aggarwal A (2015) Molecular analysis of gastric cancer identifies subtypes associated with distinct clinical outcomes. *Nat Med* **21**, 449–456.
5. Erickson LA and Lloyd RV (2004) Practical markers used in the diagnosis of endocrine tumors. *Adv Anat Pathol* **11**, 175–189.
6. Fujimura Y, Kurihara K, Ida M, Kosaka R, Miura D, Wariishi H, Maeda-Yamamoto M, Nesumi A, Saito T, Kanda T, Yamada K and Tachibana H (2011) Metabolomics-driven nutraceutical evaluation of diverse green tea cultivars. *PLoS One* **6**, e23426.
7. Fukui H, Takada M, Chiba T, Kashiwagi R, Sakane M, Tabata F, Kuroda Y, Ueda Y, Kawamata H, Imura J and Fujimori T (2001) Concurrent occurrence of gastric adenocarcinoma and duodenal neuroendocrine cell carcinoma: a composite tumour or collision tumours ? *Gut* **48**, 853–856.
8. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun Y, Jacobsen A, Sinha R, Larsson E, Cerami E, Sander C and Schultz N (2013) Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal* **6**, l1.
9. Giraudo CG, Eng WS, Melia TJ and Rothman JE (2006) A clamping mechanism involved in SNARE-dependent exocytosis. *Science* **313**, 676–680.
10. Glynn D, Gibson HE, Harte MK, Reim K, Jones S, Reynolds GP and Morton AJ (2010) Clorgyline-mediated reversal of neurological deficits in a Complexin 2 knockout mouse. *Hum Mol Genet* **19**, 3402–3412.
11. Ion Reporter Software User Guide: Tumor-Normal pair workflow (URL: https://ionreporter.thermofisher.com/ionreporter/help/GUID-76DAA3C5-573B-4446-B467-A7185DF28C6D.html)
12. Ishida M, Sekine S, Fukagawa T, Ohashi M, Morita S,

Taniguchi H, Katai H, Tsuda H and Kushima R (2013) Neuroendocrine carcinoma of the stomach: morphologic and immunohistochemical characteristics and prognosis. *Am J Surg Pathol* **37**, 949–959.

13. Japanese Gastric Cancer Association (2011) Japanese classification of gastric carcinoma: 3rd English edition. *Gastric Cancer* **14**, 101–112.
14. Komatsu H, Kakehashi A, Nishiyama N, Izumi N, Mizuguchi S, Yamano S, Inoue H, Hanada S, Chung K, Wei M, Suehiro S and Wanibuchi H (2013) Complexin-2 (CPLX2) as a potential prognostic biomarker in human lung high grade neuroendocrine tumors. *Cancer Biomark* **13**, 171–180.
15. Kotelnikova EA, Pyatnitskiy M, Paleeva A, Kremenetskaya O and Vinogradov D (2016) Practical aspects of NGS-based pathways analysis for personalized cancer science and medicine. *Oncotarget* [Epub ahead of print]
16. Kubota T, Ohyama S, Hiki N, Nunobe S, Yamamoto N and Yamaguchi T (2012) Endocrine carcinoma of the stomach: clinicopathological analysis of 27 surgically treated cases in a single institute. *Gastric Cancer* **15**, 323–330.
17. Landrum MJ, Lee JM, Riley GR, Jang W, Rubinstein WS, Church DM ann Maglott DR (2014) ClinVar: public archive of relationships among sequence variation and human phenotype. *Nucleic Acids Res* **42**, D980–985.
18. Nishikura K, Watanabe H, Iwafuchi M, Fujiwara T, Kojima K and Ajioka Y (2003) Carcinogenesis of gastric endocrine cell carcinoma: analysis of histopathology and p53 gene alteration. *Gastric Cancer* **6**, 203–209.
19. Robinson JT, Thorvaldsdottir H, Winckler W, Guttman M, Lander ES, Getz G and Mesirov JP (2011) Integrative genomics viewer. *Nat Biotechnol* **29**, 24–26.
20. Scardoni M, Vittoria E, Volante M, Rusev B, Bersani S, Mafficini A, Gottardi M, Giandomenico V, Malleo G, Butturini G, Cingarlini S, Fassan M and Scarpa A (2014) Mixed adenoneuroendocrine carcinomas of the gastrointestinal tract: targeted next-generation sequencing suggests a monoclonal origin of the two components. *Neuroendocrinology* **100**, 310–316.
21. Scherczinger CA, Bourke MT, Ladd C and Lee HC (1997) DNA extraction from liquid blood using QIAamp. *J Forensic Sci* **42**, 893–896.
22. Seruca R, Santos NR, David L, Constancia M, Barroca H, Carneiro F, Seixas M, Peltomaki P, Lothe R and Sobrinho-Simoes M (1995) Sporadic gastric carcinomas with microsatellite instability display a particular clinicopathologic profile. *Int J Cancer* **64**, 32–36.
23. Sherry ST, Ward MH, Kholodov M, Baker J, Phan L, Smigielski EM and Sirotkin K (2001) dbSNP: the NCBI database of genetic variation. *Nucleic Acids Res* **29**, 308–311.
24. Solcia E, Arnold R amd Capella C (2010) Neuroendocrine neoplasms of the stomach. *WHO Classification of Tumours of the Digestive System. Fourth ed.* 64–68.
25. Tang J, Maximov A, Shin OH, Dai H, Rizo J and Sudhof TC (2006) A complexin/synaptotagmin 1 switch controls fast synaptic vesicle exocytosis. *Cell* **126**, 1175–1187.
26. Torrent Suite v4.4.3 User and Admin Guide (URL: https://ioncommunity.thermofisher.com/docs/DOC-9305)
27. UniProt Consortium (2015) UniProt: a hub for protein information. *Nucleic Acids Res* **43**, D204-D212.
28. Wang K, Kan J, Yuen ST, Shi ST, Chu KM, Law S, Chan TL, Kan Z, Chan AS, Tsui WY, Lee SP, Ho SL, Chan AK, Cheng GH, Roberts PC, Rejto PA, Gibson NW, Pocalyko DJ, Mao M, Xu J and Leung SY (2011) Exome sequencing identifies frequent mutation of ARID1A in molecular subtypes of gastric cancer. *Nat Genet* **43**, 1219–1223.
29. Wang K, Yuen ST, Xu J, Lee SP, Yan HH, Shi ST, Siu HC, Deng S, Chu KM, Law S, Chan KH, Chan AS, Tsui WY, Ho SL, Chan AK, Man JL, Foglizzo V, Ng MK, Chan AS, Ching YP, Cheng GH, Xie T, Fernandez J, Li VS, Clevers H, Rejto PA, Mao M and Leung SY (2014) Whole-genome sequencing and comprehensive molecular profiling identify new driver mutations in gastric cancer. *Nat Genet* **46**, 573–582.
30. Wishart D, Knox C, Guo A, Shrivastava S, Hassanali M, Stothard P, Chang Z and Woolsey J (2006) DrugBank: a comprehensive resource for in silico drug discovery and exploration. *Nucleic Acids Res* **34**, D668-D672.
31. Yamaguchi K, Urakami K, Ohshima K, Mochizuki T, Akiyama Y, Uesaka K, Nakajima T, Takahashi M, Tamai S and Kusuhara M (2014) Implementation of individualized medicine for cancer patients by multiomics-based analyses-the Project HOPE. *Biomed Res (Tokyo)* **35**, 407–412.

	NEC						Ad													
	2	3	4	5	6	7	1	2	3	4	5	7	8	9	10	11	12	13	14	
MYT1	6.2	5.2	4.0	5.5	7.0	5.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.98
CHRNB2	7.3	5.0	5.3	6.3	3.9	7.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.2	0.96
ZNF775	3.0	2.5	1.6	2.7	3.0	1.6	0.0	1.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.94
DRD2	7.3	7.1	4.0	6.1	6.2	5.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.5	0.94
AP3B2	6.4	5.0	3.6	4.2	5.2	3.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.9	0.92
CPLX2	4.0	6.0	1.4	4.0	6.2	4.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.92
LOC284669	3.8	2.5	1.7	2.7	3.6	2.7	1.7	0.0	0.0	1.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.90
KCNH6	6.4	2.9	3.2	3.3	3.0	7.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.90
VWA5B2	2.5	3.4	1.9	2.1	2.6	4.9	-3.4	-2.2	-1.5	0.0	-3.9	-3.1	0.0	-2.4	-2.1	0.0	-2.4	-1.8	-3.5	0.89
STRA6	2.4	8.7	6.2	6.6	8.8	5.5	0.0	0.0	0.0	4.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.89
BUB3	1.6	1.6	0.0	1.2	1.3	1.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.88
GOLGA7B	3.6	6.2	3.0	4.0	4.4	4.5	1.8	0.0	0.0	0.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.9	0.87
RUNDC3A	4.4	5.5	2.3	2.6	3.7	4.0	0.0	0.0	0.0	0.0	2.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.0	0.87
TMEM198	3.0	2.2	2.8	2.3	2.1	3.2	0.0	1.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-2.0	0.0	1.7	0.87
SMC3	1.3	1.8	0.0	1.2	1.4	1.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.87
BSN	4.6	3.7	1.2	4.0	1.4	5.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.87
SLC36A4	1.7	1.5	2.3	1.8	1.5	2.2	1.3	0.0	0.0	0.0	1.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.87
NOL4L	4.8	4.4	5.0	2.9	4.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.86
LOC100128563	1.1	1.3	1.5	1.5	3.3	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.86
AMER3	4.5	4.8	2.9	3.3	0.0	5.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.84
EXOC7	1.6	2.5	2.1	2.2	1.5	2.2	0.0	0.0	0.0	0.0	0.0	0.0	1.6	0.0	0.0	0.0	1.6	0.0	0.0	0.84
CAMK2N2	4.5	6.4	3.3	1.7	1.8	2.8	0.0	0.0	-1.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-1.2	0.0	1.1	0.84
SMYD3	1.2	1.7	1.3	1.9	3.1	1.7	0.0	0.0	0.0	0.0	1.0	0.0	-1.5	0.0	0.0	0.0	0.0	0.0	0.0	0.84
TMEM178B	4.4	4.3	3.8	4.5	5.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.2	0.84
HIP1	1.8	2.2	1.1	1.2	2.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.83
SRRM3	3.5	5.4	3.1	2.2	1.6	4.0	1.3	0.0	0.0	0.0	0.0	0.0	0.0	1.5	0.0	0.0	0.0	0.0	2.1	0.83
SYP	2.3	3.1	3.0	0.0	1.4	3.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.83
ITPR2	0.0	1.5	1.3	2.2	3.0	2.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.83
SBK1	1.5	4.7	4.2	2.7	2.2	1.5	0.0	0.0	-1.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.83
CDKAL1	0.0	2.3	2.8	1.2	2.1	3.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.82
SCN8A	2.4	3.9	0.0	2.3	1.9	3.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.4	0.0	0.0	0.0	0.81
PROX1	4.4	2.2	2.6	4.7	6.0	5.7	4.1	3.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.80
OVOS2	6.5	1.4	2.7	3.5	5.8	2.7	0.0	1.5	1.7	1.1	0.0	0.0	1.3	1.7	0.0	0.0	0.0	0.0	0.0	0.80
TEX101	5.3	1.3	0.0	4.3	5.5	2.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.80
EVA1A	5.2	3.9	4.0	5.1	3.7	3.8	0.0	0.0	0.0	0.0	4.2	0.0	0.0	0.0	0.0	0.0	0.0	3.5	0.0	0.80
HOXA13	0.0	2.1	0.0	0.0	0.0	0.0	5.9	5.2	7.7	8.0	3.2	6.3	8.1	0.0	7.3	5.5	6.4	6.6	4.7	-0.81
CEBPA	-1.2	-4.8	-2.3	-2.8	-1.6	-1.9	-1.1	1.3	2.2	1.0	1.6	2.0	2.2	0.0	0.0	0.0	1.1	0.0	0.0	-0.82
MPPE1	0.0	-1.6	-1.4	-1.3	-1.3	-1.1	0.0	0.0	1.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-0.82
ACSM3	-6.0	-4.1	-2.9	-4.4	-7.6	-4.6	-1.8	0.0	0.0	-1.0	-1.3	-1.2	0.0	-3.3	-2.2	2.5	1.5	0.0	-1.2	-0.83
KCTD1	-2.8	-1.1	-1.7	-2.4	-3.6	-2.5	0.0	-1.3	2.4	0.0	0.0	0.0	0.0	0.0	0.0	1.8	0.0	0.0	1.0	-0.83
ST6GALNAC1	-8.5	-8.9	-7.2	-10.4	-11.6	-4.0	-2.2	-3.1	-1.3	-2.7	-5.3	0.0	1.7	-2.8	-2.3	0.0	2.0	0.0	-3.2	-0.84
MOB3B	-2.7	-4.5	-2.0	-3.0	-3.4	-1.3	-1.2	-1.2	0.0	-1.3	0.0	0.0	0.0	0.0	0.0	0.0	1.1	0.0	-1.1	-0.84
NFIB	-1.4	-1.7	-1.5	-2.5	-1.5	-2.3	0.0	0.0	1.2	0.0	-1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-0.85
TMEM37	-2.8	-3.4	-3.0	-3.3	-3.7	-2.6	-2.5	-1.1	0.0	1.1	0.0	0.0	0.0	-1.6	0.0	0.0	0.0	0.0	0.0	-0.88

Supplemental Figure Expression profile of 44 genes that contributed to the difference between NEC and GAD.